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(57) Abstract

A process for altering the target insect range (spectrum) of pesticidal toxins which comprises recombining *in vivo* the hypervariable regions of two genes encoding a pesticidal toxin and having enough residual homology as to be able to promote *in vivo* recombination. According to the present invention, truncated genes obtained from well known strains of *Bacillus thuringiensis* variety *kurstaki* and separated by an antibiotic resistant marker gene - or part of it - are cloned in a plasmid vector which i then introduced in a strain of *E. coli*. *In vivo* recombination between the hypervariable regions of the toxins genes reconstitutes a entire hybrid toxin gene. Polypeptides encoded by these new hybrid toxin genes have different biological activity and an altere target insect range as compared to their parental toxin.

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NEW FUNCTIONAL BACILLUS THURINGIENSIS HYBRID GENES OBTAINED BY IN VIVO RECOMBINATION

Bacillus thuringiensis is a spore-forming bacterium which, upon sporulation, produces an insecticidal parasporal crystal. Many Bacillus thuringiensis subspecies have been isolated and the vast majority of the strains which have been tested have shown specific activity only against larvae of Lepidopteran insects such as Manduca sexta, Heliotis virescens and Trichoplusia ni. Of the Lepidopterans, not all are equally sensitive to Bacillus thuringiensis For example, Spodoptera species tend to be relatively insensitive.

The Lepidopteran specific Bacillus thuringiensis strains have been categorized according to flagellar serotype, crystal serotype as well as activity spectrum against various insects (Dulmage, 1981). Among the better studied varieties of Lepidopteran specific Bacillus thuringiensis are B.t. kurstaki HD1 which is the strain used in "Dipel", B.t. HD 73, B.t. dendrolimus, B.t. sotto, B.t. Berliner.

In recent years, new types of Bacillus thuringiensis with novel insecticidal specificities have been discovered. B.t. israelensis is toxic to larvae of several Dipteran species (mosquitoes and black flies), but not to Lepidopteran larvae (Goldberg and Margaht, 1977). More recently, two Coleopteran specific strains, B.t. tenebrionis and B.t. San Diego, which later were shown to be the same strain (Krieg et al., 1987), have been described (Krieg et

al., 1983, Krieg et al., 1984; Herrnstadt et al., 1986). These strains have shown activity against Colorado potato beetle and other Coleopteran pests.

The entomopathogenic activity of the _Bacillus thuringiensis_ parasporal crystal is due to its composition: in the case of the _Bacillus thuringiensis_ subspecies specifically active against Lepidopteran larvae, it is composed of 130 to 160 Kdal protoxin polypeptides. Different subspecies and often individual strains of the same subspecies produce endotoxins having a characteristic spectrum of insect toxicity (Whiteley and Schnepf, 1986; Andrews et al., 1987).

For many years _Bacillus thuringiensis_ has served as the basis of successful biological insecticides. To produce these insecticides, _Bacillus thuringiensis_ is fermented until spores and crystals are obtained. The mixture of spores and crystals is then formulated to allow effective application on crop plants..

Current, traditional _Bacillus thuringiensis_ products are in fact an example of classical industrial microbiology. These products are created through such traditional microbiological practices as strain isolation and improvement, and fermentation optimization.

Two features of _Bacillus thuringiensis_ have made it a popular and useful insecticide. First, _Bacillus thuringiensis_ is considered extremely safe: it is harmless to humans, animals and useful insects. Second, _Bacillus thuringiensis_ is a highly specific ins cticide: most strains of _Bacillus thuringiensis_ show toxicity to only a single order of insects

(Lepidopt ra or Col optera or Diptera), as indicated above.

These features have also made Bacillus thuringi nsis an attractive target for biotechnology and recently the new tools of this modern technique, such as gene cloning and DNA sequencing, have begun to be applied to Bacillus thuringiensis, thus creating novel Bacillus thuringiensis pesticidal proteins with either more specificity, more toxic activity or an altered range of toxicity for the host insect.

The insecticidal activity of Bacillus thuringiensis resides in the parasporal crystal (Angus, 1954). Intact crystals can be isolated from sporulated cultures of Bacillus thuringiensis by density gradient centrifugation and these isolated crystals of the Lepidopteran specific Bacillus thuringiensis variety kurstaki were shown to be composed by protein subunits of approximately 130,000 daltons (Bulla et al. 1977). In some strains such as Bacillus thuringiensis variety kurstaki HD-73 there appears to be a single protein subunit, while in other strains such as Bacillus thuringiensis HD1 there appear to be two or more very similar proteins in the crystal (Wilcox et al., 1986).

The 130.000 dalton protein is considered to be a protoxin because it is toxic to larvae only after injestion, but not after injection. The protoxin can be converted to the active toxin by digestion with proteas s; and it has also been possible to isolate proteolytic fragments of the protoxin which retain full toxic activity.

Bulla et al., 1981, found that a 68,000 dalton toxin

fragment could be derived from Bacillus thuringiensis kurstaki crystals upon prolonged incubation of the solubilized protein.

Crystals of the Coleopteran specific Bacillus thuringiensis strains also appear to be composed of a single protein subunit, but of a much smaller size than the Lepidopteran protoxin.

Bernhard, 1986, isolated a 68,000 dalton protein from crystals of Bacillus thuringiensis tenebrionis, and Herrnstadt et al., 1986, observed a 64,000 dalton protein from Bacillus thuringiensis San Diego crystals. These isolated proteins are toxic upon ingestion by sensitive Coleopterans.

The crystal protein of the Lepidopteran specific Bacillus thuringiensis var. kurstaki strain, produced during the sporulation period, is also known as - endotoxin, and around 20-30% of the cell protein synthetizing activity during sporulation is devoted to the production of this toxin.

Much work has recently been directed to the isolation and characterization of genes encoding Bacillus thuringiensis toxins. The analysis of such cloned genes has already yielded important insights into toxin structure and function: it has been shown for example that genes for the crystal proteins are located on large plasmid in addition to chromosomal DNA.

Several groups have reported cloning genes for Lepidopt ran specific toxins. Most of these genes have been cloned in E. coli, either utilizing antibodies to purified toxin to detect expression of the toxin, or utilizing synthetic oligonucleotide

probes bas d on the toxin aminoacid sequence to detect toxin genes by hybridation. The genes cloned include s veral genes from Bacillus thuringiensis kurstaki HD1 (Schnepf and Whiteley, 1981; Held et al., 1982; Watrud et al., 1985; Shivakumar et al., 1986; Thorne et al., 1986), and genes from Bacillus thuringiensis kurstaki HD 73 (Adang et al., 1985), from Bacillus thuringiensis sotto (Shibano et al., 1985), Bacillus thuringiensis Berliner (Klier et al., 1982; Wabiko et al., 1986), Bacillus thuringiensis aizawa (Klier et al., 1985) and Bacillus thuringiensis thuringiensis (Honigman et al., 1986).

In general these genes have been shown to express toxin in E. coli and extracts of E. coli harbouring these genes are toxic to Lepidopteran larvae.

The cloned toxin genes have been used as molecular probes to determine the toxin gene number and type of many Lepidopteran active Bacillus thuringiensis strains (Kronstad et al., 1983). This analysis has shown that, while some strains (e.g. Bacillus thuringiensis kurstaki HD73) contain only a single toxin gene, many other strains contain multiple genes. Bacillus thuringiensis kurstaki HD-1 (the Dipel strain) has three distinct toxin genes (Wilcox et al., 1986).

DNA sequences and derived aminoacid sequences of the toxin proteins have been determined for several of these genes. All the genes encode proteins of between 1156 and 1178 aminoacids which are largely homologous. In some cases genes isolated from strains which had been considered distinct varieti s hav been found to be nearly identical. For example, the

Bacillus thuringiensis kurstaki HD-1 gene sequenced by Schnepf et al (1985) is nearly identical to the Bacillus thuringiensis sotto gene of Shibano et al. (1985). Similarly, a Bacillus thuringiensis Berliner gene (Wabiko et al., 1986) is nearly identical in sequence to another Bacillus thuringiensis kurstaki HD-1 gene. On the other hand, a third gene from Bacillus thuringiensis kurstaki HD-1 (Thorne et al. 1986) is clearly different in sequence from the two mentioned above.

Cloning and sequencing of the structural genes for the protoxin production from distinct strains of Bacillus thuringiensis kurstaki have, therefore, revealed that different related genes are responsible for the synthesis of the crystal protein toxin. These differences are evident not only among genes from different strains, but also among the multiple copies of the protoxin gene in the same strain (Andrews et al., 1987). The kurstaki HD-1 Dipel protoxin gene (Schnepf et al. 1985) and the kurstaki HD-73 protoxin gene (Adang et al., 1985) show an homology of 85% at the primary DNA sequence level. K-1 type and K-73 type crystals show distinct toxic activity against different insect species (Jaquet et al., 1987).

More particularly, with reference to the present invention, comparisons of DNA sequences encoding the crystal toxin from distinct strains of Bacillus thuringiensis have revealed the existence of both conserved and variable r gions. A close look to these variable regions has shown that changes are not distributed randomly over the whole gene coding for the crystal toxin, but that differences among genes

are clustered in a hypervariable region (Geiser et al. 1986).

Only few changes or no differences have been shown at the N-terminus and the C-terminus of the crystal protein genes. In fact, from the N-terminus all genes are nearly identical for approximately the first 330 aminoacids. Similarly, from about aminoacid 600 through the C-terminus the genes are largely the same.

Optimal alignment of the DNA sequences and of the deduced polypeptide sequences of these two genes and of other <u>Bacillus thuringiensis</u> genes reveals that the differences are clustered in the amino terminal halves of the molecules i.e. between aminoacid residues 280 and 640 in the case of HD-1 Dipel and HD-73 genes. This region, as hereabove mentioned, is defined as hypervariable region since it shows the maximum of variability (Geiser et al., 1986; Wabiko et al., 1986; Andrews et al., 1987).

On the basis of published sequences there are at least four distinct types of Lepidopteran toxins which differ substantially in this central region. Generally, the hypervariable region is, as mentioned above, in the first half of the protoxin sequence. This hypervariable region might be the result of intramolecular recombination mechanisms between very similar, but distinct genes. Moreover, the clustering of the variable subdomains in exact regions of the crystal protein toxin, strongly suggests that the exchange of hyper variable regions betwe n genes may have caused the larg variability of biological activities of different <u>Bacillus</u> <u>thuringiensis</u>

crystal protein toxins. This variability is important in conferring toxic diversity and/or target insect range diversity among different *Bacillus thuringiensis* subspecies. The residual homology of the hypervariable region (in the case of HD-1/Dipel and HD-73 reduced to about 60% at the DNA level) should be sufficient to promote *in vivo* recombination.

A similar approach has been utilized to generate, in *Escherichia coli*, recombinants between human leukocyte interferon genes (EP 141484) and for *Bacillus* alpha amylase genes (Rey et al., 1986).

Deletion analysis of Lepidopteran toxin genes has allowed the construction of much smaller proteins with full toxicity. These truncated genes show full insecticidal activity only when the entire hypervariable region is present.

This analysis has been carried out for the *Bacillus thuringiensis kurstaki* HD-73 gene (Adang et al., 1985), the *Bacillus thuringiensis sotto* (Shibano et al., 1985), two genes from *Bacillus thuringiensis kurstaki* HD-1 (Schnepf and Whiteley, 1985) and a Bacillus thuringiensis Berliner gene (Wabiko et al, 1986).

Since the biological activity and the action range of the crystal toxin proteins seem to be associated to the hypervariable region sequence, and since there is a specific need to produce new specific *Bacillus thuringiensis* toxin, the applicant devised, according to the present invention, a novel way of generating new hybrid genes and corresponding hybrid toxin proteins, with potential different toxic sp cificity, by modifying the hypervariable regions of the genes

encoding the crystal protein toxins.

Modification of the hypervariable r gions of the crystal protein encoding genes could be don by site-specific mutagenesis, a technique consisting basically in introducing specific nucleotide mutations, either substitutions or deletions, in the crystal toxin hypervariable gene region, in order to obtain aminoacid substitutions in the polypeptide chain encoded by such mutagenized DNA sequence.

This technique, nevertheless, is in general very useful and efficient only if the molecular mechanism of action of the protein to be modified is known. The tridimensional structure of the protein should be known as well to be able to forecast what could be the consequences of the specific aminoacid substitutions or deletions in the protein to be modified. With reference to the Bacillus thuringiensis crystal toxin protein there are no data available as to its tridimensional structure and this is due at least to two main reasons:

1) It is quite difficult to obtain crystals of the toxin protein which can be used in a crystallographic analysis.

2) Last but not least, the size of the polypeptide chain (135,000 dalton) would make the crystallographic analysis very laborious.

As a consequence, the site-specific mutagenesis does not seem to be very useful in providing an easy and efficient means to obtain new Bacillus thuringiensis crystal toxins.

Another alternative approach could b a mutagenesis conducted at random on a Bacillus thuringiensis

crystal toxin cloned gene, but this method, lacking in specificity, does not seem to be useful to obtain new toxins characterized by insecticidal activities, since mutations introduced in the toxin gene according to mutagenesis conducted at random only affect a limited number of aminoacid residues.

There is therefore a specific need to provide an easy and efficient method capable of producing new Bacillus thuringiensis crystal toxin by modifying the hypervariable region of the crystal toxin gene.

Accordingly, the present invention, by means of an *in vivo*-recombination process, provides a potentially unlimited number of new hybrid genes coding for new corresponding hybrid crystal protein toxins having either different insecticidal activities and/or an alterated target insect range.

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The present invention concerns new hybrid Bacillus thuringiensis genes, obtained by in vivo recombination, encoding new corresponding Bacillus thuringiensis hybrid crystal protein toxins having ith r different insecticidal aciviti s and/or an

altered insect host range.

The new/ hybrid *Bacillus thuringiensis* genes object of the present invention ar obtained, as above mentioned, by means of *in vivo* recombination of the hypervariable region present in the *Bacillus thuringiensis* genes coding for the *Bacillus thuringiensis* crystal protein toxins.

The present invention also comprises new polypeptides, e.g. new hybrid crystal protein toxins obtained by. *in vivo* recombination of the hypervariable region of two genes coding for the *Bacillus thuringiensis* crystal protein toxins.

The two genes to be recombined *in vivo* can be:

a) derived from natural *Bacillus thuringiensis* strains

b) they can be the products of a previous *in vivo* recombination event.

Another embodiment of the present invention refers to a novel process of production of new hybrid pesticidal toxins, in particular *Bacillus thuringiensis* crystal protein toxin, by *in vivo* recombination of the hypervariable regions of genes coding for a pesticidal toxin, said genes having enough residual homology to be able to recombine *in vivo*.

In a preferred embodiment of this invention the two hypervariable regions to be recombined *in vivo* come from two different genes of *Bacillus thuringiensis kurstaki* and more particularly one gene is the crystal toxin encoding gene from *Bacillus thuringiensis kurstaki* HD-1 Dipel (Gene HD-1) and the other gene is th crystal toxin encoding gene from

Bacillus thuringiensis kurstaki HD-73 (Gene HD-73). According to the present invention the *in vivo* r combination process applies to crystal protein encoding genes isolated from the following strains:

Bacillus thuringiensis alesti
- aizawai
- canadensis
- dakota
- darmstadiensis
- dendrolimus
- entomocidus
- finitimus
- fowleri
- galleriae
- indiana
- israelensis
- Keniae
- kurstaki
- kyushuensis
- morrisoni
- ostriniae
- pakistani
- San Diego
- sotto
- tenebrionis
- thompsoni
- thuringiensis

Bacillus thuringiensis tolworthi
- toumanoffi
- wuhanensi

Th present invention also ref rs to plasmid vectors which contain two genes encoding a p sticidal protein

toxin or a part thereof, said gen s having enough residual homology to be able to recombine _in vivo_.

In an embodiment of the present invention these genes are the genes encoding the _Bacillus thuringiensis_ crystal protein toxin.

In a preferred embodiment of the present invention these genes are the genes encoding the _Bacillus thuringiensis_ variety _kurstaki_ crystal protein toxin and in a more preferred embodiment of the present invention these genes are the genes encoding the _Bacillus thuringiensis_ variety _kurstaki_ HD-1 Dipel crystal protein toxin (gene HD-1) and the _Bacillus thuringiensis_ variety _kurstaki_ HD-73 crystal protein toxin (gene HD-73).

These plasmid vectors containing the _Bacillus thuringiensis_ DNA sequences to be recombined _in vivo_ (for the reasons explained below they might be called "father plasmid vectors") represent a source of a potentially unlimited number of plasmid vectors ("son plasmid vectors") wherein the _Bacillus thuringiensis_ DNA sequences have recombined _in vivo_ and now these new hybrid DNA sequences encode new _Bacillus thuringiensis_ hybrid crystal toxins.

The present invention also refers to plasmid vectors ("son plasmid vectors" as above mentioned) containing new _Bacillus thuringiensis_ hybrid DNA sequences, resulting from _in vivo_ recombination of two _Bacillus thuringiensis_ genes encoding the crystal protein toxin wherein thes new hybrid DNA sequences encode n w _Bacillus thuringiensis_ hybrid crystal toxins having either differ nt insecticidal activities and/or an altered insect host range.

Objects of the present invention also are expression vectors containing the new hybrid Bacillus thuringiensis DNA sequences obtained according to this inventions and regulatory functions (like promoters, attenuators, ribosome binding sites, specific SHINE-DALGARNO sequences, stop codons, enhancers) which allow a very high expression of said new hybrid Bacillus thuringiensis DNA sequences and therefore a high production of their corresponding hybrid polypeptides.

Expression controlling sequences useful in expressing the new hybrid B. thuringiensis DNA sequences of this invention include, but are not limited to, the lac system, trp system, the major operator and promoter regions of phage λ, the control regions of fd-coat protein, the β- lac system, the TAC system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses or combination thereof.

Another embodiment of the present invention refers to host cells transformed by plasmid vectors as here above described.

Hosts useful for preparation of the hybrid DNA sequences of the present invention by in vivo recombination include various strains of E. coli, Pseudomonas, B. subtilis, B. thuringiensis, Agrobacterium, yeasts.

For the transformation, for obtaining the hybrid Bacillus thuringiensis DNA sequences of the present invention, host cells can be r combination-proficient, $recA^+$.

Moreover, th in vivo recombination process of

Bacillus thuringiensis DNA sequences encoding crystal toxin protein occurs in host cells rec A$^-$, rec A$^-$ strains can also be used.

the use of recA$^-$ strains for in vivo recombination provides a further differentiation from the one described by Weissmann and Weber in EP-141484.

In a preferred embodiment of the present invention Escherichia coli host cells have been used.

Two father plasmid vectors called pT173 and pGEM173 were transformed in Escherichia coli HB 101, so giving rise to strains I-879 and I-878, filed at Paris Pasteur Institute on June 28, 1989. The skilled persons in the art can understand that several other host cells may be used.

Host cells useful for the expression of the hybrid Bacillus thuringiensis DNA sequences of the present invention are:

E. coli

Bacillus different species

Bacillus thuringiensis

Agrobacterium

Yeasts

Baculoviruses

Rhizobium

The present invention also comprises the use of the new hybrid Bacillus thuringiensis DNA sequences, object of this invention, for the preparation of new plasmid vectors containing thes new hybrid Bacillus thuringiensis DNA sequences, wherein said plasmid vectors are used to transform plant cells.

New hybrid Bacillus thuringi nsis DNA sequences of th present invention coding for new hybrid Bacillus

thuringiensis crystal prot in toxins can in fact be engineered into plant cells to yield insect resistant plants.

The methodology for engineering plant cells is well established (see Nester E.W., Gordon M.P., Amasino R.M. and Yanofsky M.F., Ann. Rev. Plan. Physiol. 35:387-399, 1984;and EP 142 924).

Plant cells transformed by these plasmid vectors are also within the scope of the present invention.

The present invention comprises as well transgenic plants containing new hybrid Bacillus thuringiensis DNA sequences as produced according to this invention.

In addition to be used to transform plant cells, the new hybrid Bacillus thuringiensis DNA sequences of the present invention can be introduced into microorganisms capable of occupying, surviving and proliferating in the phytosphere of plants according to the procedures disclosed in EP-0200344.

The present invention comprises as well muteins of the hybrid Bacillus thuringiensis protein sequences of this invention, wherein these muteins have been obtained by standardized genetic engineering techniques (like site-specific mutagenesis, random mutagenesis, glycosilation) and their activity is reconducible to the insecticidal activity of their parent hybrid Bacillus thuringiensis crystal toxin proteins.

The present invention also comprises new p sticidal compositions containing new hybrid Bacillus thuringiensis polypeptides obtained according to this invention, in combination with suitable eccipients,

adjuvants and aggregants etc. These compositions are pr par d by intimately and uniformly mixing the new hybrid <u>Bacillus thuringiensis</u> polypeptides of the present invention with suitable finely divided diluents, fillers, eccipients, disintegrating agents and the like.

As to the process of obtaining <u>in vivo</u> recombination of DNA homologous sequences we referred to a method developed by Weber and Weissman (1983) and EP-141484.

The methods of their invention are characterized by the steps of a) preparing a DNA fragment or concatemer thereof, said fragment comprising in sequence one of the parental DNA sequences from which the hybrid DNA is to be derived, an intact replicon such that the DNA fragment may be replicated in a host cell, and the other parental DNA sequence from which the hybrid DNA sequence is to be derived, the two parental DNA sequences having sufficient homology to promote their recombination <u>in vivo</u>; and b) selecting host cells that have been transformed with the desired hybrid DNA sequence and isolating said hybrid DNA sequence from them. Selection of the desired host cells may be facilitated by having each parental DNA sequence associated with a different antibiotic resistance marker, and growing the transformed host cells on agar plates containing both antibiotics.

The hybrid DNA sequences produced by these methods clearly enable the production of novel hybrid polypeptide having a variety of uses and biological activities.

According to EP 141484, th s methods were originally

used to produce new hybrid interferons genes by in vivo recombination of two interferon genes α-1 and α-2 having partial sequence homology (80%).

Thus DNA structures consisting of plasmid vector sequences flanked by the α-2 interferon gene on the one side and a portion of the α-1 interferon gene on the other were transfected into E. coli host cells. Appropriate resistance markers allowed the isolation of colonies containing circular plasmides which arose by in vivo recombination between the partly homologous interferon gene sequences. In the plasmid vectors different recombinant genes were identified, all of them encoding for new hybrid interferon not easily accessible by traditional recombinant DNA techniques. This method, according to EP 141484, should be generally applicable to the formation of recombinants between not too distantly related genes. But, while according to the method described by Weissmann and Weber in EP-141484 host cells should be recombination-proficient $recA^+$, we have recently found that in vivo recombination of the hypervariable regions of Bacillus thuringiensis genes encoding the Bacillus thuringiensis crystal protein toxin occurs in host cells as well that are $recA^-$. Furthermore the experimental process of the applicant thanks to the use of father plasmid vector, is shorter, safer and less difficult than that described by Weber and Weismann in EP-141484.

With a techniqu similar to that disclosed in Ep-141484 Rey et al., 1986, obtained r combinant -amylases by in vivo recombination between the genes coding the B. licheniformis α-amylase and the

homologous gene coding the B. stearothermophilus α-amylase.

Another way of producing novel toxins or altering the insect host range of Bacillus thuringiensis toxins is provided by a method, described in EP 228838 herein enclosed as a reference, which comprises recombining in vitro the variable regions of two or more δ-endotoxin genes.

Specifically exemplified in EP 228838 is the recombining of variable regions to two Bacillus thuringiensis kurstaki sequences, in particular HD-1 and HD-73, to produce chimeric Bacillus thuringiensis δ-toxins with altered ranges as compared to the toxins produced by their parent DNA.

Variable regions, as used in that patent application, refers to non-homologous regions of two or more B.t. δ-DNA sequences which upon in vitro recombination yields a DNA sequence encoding a new δ-endotoxin with an altered insect host range. According to the method described in EP 228838, two Bacillus thuringiensis gene showing partial homology are recombined in vitro by first cutting and then religating with restriction enzymes specific Bacillus thuringiensis DNA sequences in order to obtain recombination of the non-homologous regions.

However, even if this method is quite specific and allows production of new Bacillus thuringiensis toxins, it suffers as well of a considerable drawback since, according to an in vitro recombination process, only a limited amount of hybrid proteins can be produced. The method of EP 228838 requires in fact the identification of specific restriction sites in

both the genes to be recombined, while the method of the pres nt invention, wher in recombination is carried out <u>in vivo</u>, allows the production of a potentially unlimited number of hybrid <u>Bacillus thuringiensis</u> toxins.

The disclosure of the present invention is intended to be read in conjunction with the references cited which are set forth in the appended bibliography.

The following examples which illustrate procedures, including the best mode to practice the invention, should not be considered limiting. The examples are for illustration purpose and intended to describe this invention so that it may be clearly understood.

The present invention provides new hybrid <u>Bacillus thuringiensis</u> DNA sequences obtained by <u>in vivo</u> recombination of two or more different genes encoding <u>Bacillus thuringiensis</u> crystal protein toxins.

According to the present invention, therefore, we have subcloned in plasmid vectors two truncated genes of <u>Bacillus thuringiensis</u> variety <u>kurstaki</u>.

The source of the HD-1 Dipel gene portion was the plasmid pESAC, a derivative of pES1 (ATCC 31995) described by Schnepf and Whiteley (1981). The HD-73 gene portion derived from pJWK20 plasmid (ATCC 31997) described by Kronstad and Whiteley, 1984.

pBS19 is an <u>E. coli</u> - <u>B. subtilis</u> shuttle vector derived from pBS42 (Wells et al., 1983; Greg Gray, unpubl.).

The gene obtained from plasmid pES1 of <u>Bacillus thuringiensis</u> variety <u>kurstaki</u> HD-1 (gene HD-1 Dipel) consisted in the promoter, the 5' coding sequence and the whole hypervariable coding region.

The second gene (gene HD-73) obtained from plasmid pJWK20 (Adang et al., 1985) of Bacillus thuringiensis variety kurstaki HD-73 was truncated in the 5' coding region and consisted, therefore, of the hypervariable region and the 3' terminal coding sequence.

These two genes, gene HD-1 Dipel and gene HD-73, are among those isolated from Bacillus thuringiensis variety kurstaki showing the greatest differences at the level of the nucleotide sequence of the hypervariable region. Crystal protein toxins produced by these two different bacterial serotypes Bacillus thuringiensis kurstaki HD-1 and Bacillus thuringiensis kurstaki HD-73 show different insecticidal activity for different species of Lepidopterans.

According to the present invention the two truncated genes here above described have been cloned into plasmid pBS19 which contains a gene capable of conferring the chloramphenicol resistance phenotype. Between the two cloned Bacillus thuringiensis variety kurstaki genes, namely gene HD-1 Dipel and gene HD-73, a marker gene coding for tetracycline resistance was inserted. The result of this construction is plasmid vector pT173 which is shown in Figure 1.

Another plasmid, corresponding to plasmid pT173 and called pGEM 173 (10.8 Kb) is shown in Figure 2.

This plasmid has been obtained inserting a truncated fragment of both the Bacillus thuringiensis kurstaki HD-1 gene and the Bacillus thuringiensis kurstaki HD-73 gene in the plasmid vector pGEM4Z (Promega, Madison, WI, USA Plasmid pT173, once introduced in Escherichia coli cells synthetizes a polypeptide of 65

Kd which is recognized by specific antibodies for the <u>Bacillus</u> <u>thuringiensis</u> crystal toxin protein. This polypeptide has been identified as the product of <u>Bacillus</u> <u>thuringiensis</u> variety <u>kurstaki</u> HD-1, which, as here above described, is truncated downstream of the hypervariable region. The partial homology between the two hypervariable regions of gene HD-1 Diepel and gene HD-73 (at nucleotide sequence level this homology is 62,2%) should be sufficient to be able to promote an <u>in</u> <u>vivo</u> recombination process of the two truncated genes.

It is clear that since this is an <u>in</u> <u>vivo</u> recombination process, plasmid vectors like plasmid pT173 can produce an unlimited number of different recombinant hybrid <u>Bacillus</u> <u>thuringiensis</u> DNA sequences encoding for their corresponding hybrid <u>Bacillus</u> <u>thuringiensis</u> polypeptides having either different insecticidal activity and/or an altered target insect range.

Plasmid pT173, therefore, has been transformed in suitable <u>Escherichia</u> <u>coli</u> host cell.

In one embodiment of the present invention, these <u>Escherichia</u> <u>coli</u> host cells are recombination proficient rec A^+, but according to some more recent results, <u>in</u> <u>vivo</u> recombination of <u>Bacillus</u> <u>thuringiensis</u> DNA sequences occurs also in cells which are rec A^-.

The recombination defici nt rec A^- cells (instead of recombination proficient rec A^+) have the advantage that the hybrid DNA is not rearranged. In this way accuracy of recombination is obtained at the DNA level, avoiding casual rearrangment and then the

occurence of unexpected and unwanted sequences.

After many generations plasmid DNA is extracted and digested with the restriction enzyme Nru I.

Since recombinant plasmid carrying new Bacillus thuringiensis hybrid DNA sequences obtained upon in vivo recombination have lost the DNA restriction site recognized by Nru I (as it will be explained in a more detailed way further on), treatment with the endonuclease Nru I allows selection of plasmids which have recombined in vivo.

These recombinant plasmids, being the only ones still circular after the Nru I treatment, will be able successively to transform E. coli cells producing their colonies which will be resistant to chloramphenicol.

The same procedure applies to plasmid pGEM 173, with the only difference that E. coli transformants are selected for resistance to ampicillin instead of chloramphenicol.

According to the method hereabove described, we succeeded in isolating many different recombinants whose hybrid DNA sequences are reported in Figure 6 a - e.

MATERIALS AND METHODS

BACTERIAL STRAINS

The following bacterial strains and their rec A derivative were used for transformation:

Bacterial strains

Escherichia coli strains were: HB101 (F- hsdS20 recA13 ara-14 proA2 leuB6 lacY1 galK2 rpsL20 xyl-5 mtl -1 sup E44)

JM103: (lac proAE) Δ (lac pro), thi, strA, supE,

endA, sbeB, hsdR-, F'traD36, proAB, lacI9, ZΔM15

294 endA thi pro hsdR hsdM hsm

294recA endA thi pro hsdR hsdM hsm recA

Escherichia coli host cells were made competent and transformed according to Hanahan 1985.

CULTURE MEDIA

LB medium (per liter: Difco Bacto-tryptone 10g; Difco Bacto-yeast extract 5g; NaCl 5g)

For growth of strains with plasmid pT173, tetracycline 12.5 µg/ml or chloramphenicol 10 µg/ml were added to LB medium.

For growth of strains with plasmid pGEM-173 ampicillin was used at 100 µg/ml.

PLASMID DNA EXTRACTION

Plasmid DNA preparation has been done by the alkaline lysis method adapted to larger samples and followed, for sequencing, by a PEG precipitation (Birnboim and Doly, 1979).

RESTRICTION ENZYMES, DIGESTION, ELECTROPHORESIS

Restriction enzymes were from Boehringer Mannheim GmbH II (FRG) and from Bethesda Research Laboratories (Maryland, USA).

Restriction enzymes digestion, ligation and other treatments during plasmid construction have been done following the suggested protocols of the supplier.

Restriction patterns generated from the digestion of plasmid DNA were resolved on 0,7% agarose gels (agarose from Bethesda Research Laboratories). DNA samples were electrophoresed in TBE buffer (0,3M Tris-borate pH 8.3, 2 mM EDTA) and stained with ethidium bromid . When necessary electrophoresis was performed on acrylamide gels (6%) in TBE buffer.

DNA SEQUENCE ANALYSIS

Fragm nts of DNA, originated from recombinant plasmids derived from pT173 and pGEM-173, were obtained by HindIII digestion. A band corresponding to DNA of approximately 2.9 Kb was separated by agarose gel electrophoresis, recovered by electroelution and subsequently digested with EcoRV. A band of DNA of approximately 700 bp was purified by acrylamide gel electrophoresis (6%) electroeluted and ligated to the plasmid pGEM-4Z (Promega, Madison, W1, USA) digested with SmaI and treated with phosphatase. Sequencing was performed by the chain termination method of Sanger et al. (1977) adapted to plasmid DNA (Chen and Seeburg, 1985).

Sequenase (United States Biochemical) is known, in the dideoxi-chain elongation reaction.

IMMUNOBLOTTING

The method of Towbin et al. (1974) was used to detect the crystal protein immunologically.

Proteins resolved by Sodium dodecyl sulphatepolyacrylamide gel electrophoresis were transferred electrophoretically to nitrocellulose sheets washed with 50 mM Tris hydrochloride-200 mM NaCl containing 0.1% Nonidet P-40 and then incubated with the antiserum.

After a wash with the same buffer, the nitrocellulose sheets were incubated with peroxidase-conjugated sheep anti-rabbit immunologlobulin G antiserum (United States Biochemical Co., Cleveland, Ohio).

The immuno-complexes w re then visualized in the presence of hydrogen peroxide and 4-chloro-1-naphtol as substrat s (GIBCO Laboratories, Grand Island

N.Y., Bethesda Research Laboratories, Inc. Gaithersburg, Md.)

PLASMID CONSTRUCTION

pT is a pBS19 derivative: a 1424 bp Eco RI - Ava I (with even ends in Ava I) fragment of pBR322 bearing tetracycline resistance was inserted in pBS19 digested with Eco RI and Sac I (the latter digestions followed by treatments able to obtain even ends).

pT73 is a pT derivative obtained by inserting in the Eco RI site of pT an Eco RI fragment of approximately 5400 bp obtained from pJWK20 (Fig. 1). The fragment comprises the last two thirds of the HD73 toxin gene starting from residue 1383 of the sequenced region (Adang et al., 1985). pT1 was obtained as follows: pT was digested to completion with Sma I, partially with Bam HI and ligated to a fragment of approximately 1900 bp derived from pESAC and corresponding to the first portion of HD-1 Dipel toxin gene from nucleotide residue 291 to residue 2215 of the sequenced region (Schnepf et al., 1985).

The fragment was obtained by digestion of pESAC with Hind III followed by treatment with Klenow to make it blunted and then by restriction with Bam HI.

pT173, the plasmid used for the *in vivo* construction of recombinant genes, derived from the insertion of the 2500bp Bam HI-Bam HI fragment of pT1, comprising the first part of the HD-1 Dipel gene and the last two thirds of the resistance tetracycline gene, in pT73 completely cleaved with Bam HI and dephosphorilated with pancreatic phosphatase to avoid r -insertion of the original Bam HI fragment.

Tet^R and Cm^R transformants obtained in *E. coli* 294

racA strain were examined by plasmid extraction and restriction analysis.

Plasmids pT1, pT73 and pT173 were not able to direct the synthesis of a complete endotoxine polypeptide but only of a 65-68 kdal fragment which immunoreacted with antibodies raised against the pure toxic crystal in pT1 and pT173.

The tetracycline resistant gene was inserted in inverted orientation to ensure that the HD73 truncated sequence could not be expressed from external expression control regions in pT73 and pT173.

GENERATION OF HYBRID GENES

The sequences of HD-1 Dipel and HD73 genes in pT173 share a region of homology, i.e. the last 696 bp of HD-1 Dipel sequence and the first 707 bp of the HD-73 sequence. Between these partial direct repeats there is the tetracycline resistance determinant characterized by the unique _Nru_ I site.

Figure 3 shows the protocol followed to generate the recombinant plasmids with the hybrid toxin genes.

The pT173 plasmid was introduced in a recombination proficient background by transformation of _E. coli_ 294 competent cells.

A single colony Tet^R and Cm^R has been inoculated in LB (supplemented with chloramphenicol) and grown for about 40 generations. Plasmid DNA extracted from the cells was digested with _Nru_ I. In this way the molecules not subjected recombination and having the intact tetracycline gene were lineariz d. Only circular molecules could replicate and transform the 294 _recA_ competent cells to Cm^R.

This allows selection of plasmids which have recombined *in vivo*: they have lost the restriction site recognized by the endonuclease Nru I and according to that they remain circular.

The Cm^R transformants were screened for sensitivity to tetracycline with the aim of obtaining recombinants between the partially homologous regions of the truncated toxin genes (boxed in Figure 3). In this way we expected to reconstitute an entire hybrid gene with the first third of the aminoterminal region of the HD-1 Dipel gene and two thirds from the carboxyterminal region of the HD-73 gene. The hypervariable region was expected to be a different hybrid region for each clone able to express a polypeptide immunoreacting with specific antibodies.

We isolated 13 Cm^R Tet^S clones, examined them for the production of a polypeptide immunoreacting with polyclonal antibodies raised against HD-73 toxic crystals and for the presence of recombinant plasmids (pTHy).

ANALYSIS OF THE RECOMBINANT OBTAINED

Escherichia coli cells transformed with the recombinant plasmids have been analyzed by immunoblotting to identify all the clones capable of synthesizing a polypeptide chain of 135 kd having the immunological properties of the *Bacillus thuringiensis* crystal toxin protein. The method of Towbin et al (1974) was used to detect the crystal prot in immunologically.

According to this method 10 positive clones were identified.

In order to characterize th se positive clones

identified, recombinant plasmids have been purified and their DNA was dig sted with restriction enzymes in order to obtain their restriction maps. Plasmid purification and restriction enzyme digestion have been done according to the methods indicated previously in description of the present patent application.

All the plasmids analyzed were identified as recombinant plasmids obtained by <u>in vivo</u> recombination of the hypervariable regions of the two <u>Bacillus thuringiensis kurstaki</u> genes contained either in plasmid pT173 or pGEM173.

These two "father plasmids" are in fact sources of a potentially unlimited number of new hybrid <u>Bacillus thuringiensis</u> DNA sequences obtained by <u>in vivo</u> recombination.

All these recombinant plasmids were further characterized by determining the nucleotide sequence of DNA fragments corresponding to the hypervariable regions wherein, according to the present invention, recombination has occurred.

DNA sequence analysis has been done according to the method indicated in the description of this patent application.

In figure 4 the HD-1 and the HD-73 <u>Bacillus thuringiensis</u> DNA sequences are aligned to maximize the matches (represented by a vertical line). The regions of cross-over are indicated by a box.

While most of the recombinant hybrid DNA sequences obtained and identifi d are different from each other, some of them were completely identical and we can not exclude that they might be "brothers"

resulting from a unique recombination process.

As indicated in Figure 4, the in vivo recombination process has occurred through all the hypervariable region. So far, of all the recombinant hybrid *Bacillus thuringiensis* DNA sequences analyzed, excluding those which recombined in the same region, 10 hybrids used a different region of cross-over. The DNA sequences of these 10 new recombinant hybrid *Bacillus thuringiensis* genes are reported in Figure 6 a - e.

These recombinant hybrid sequences have been called respectively: HY45, HY3, HY6, HY53, HY21, HY32, HY2, HY 127, HY 126, HY 5.

The aminoacid sequence of the proteins coded by these recombinant hybrid *Bacillus thuringiensis* genes can be easily deduced from their nucleotide sequence.

A comparison of the deduced aminoacid sequences is reported in Figure 5 which represents the optimal alignment of these polypeptide products deduced from the DNA sequence of their parental recombinant hybrid genes. According to the specific site where the recombination process has occurred, the hybrid protein corresponds in the hyper variable region either to the protein encoded by the gene HD-1 Dipel or to the protein encoded by the gene HD 73.

Two cases have been identified, HY6, HY64 and HY 127, HY 21, where, while the hybrid recombinant genes have a different nucleotide sequence, the polypeptide chains are identical. At the aminoacid level, therefore, the r al number of n w recombinants encoding hybrid *Bacillus thuringiensis* crystal protein toxins is eight. Th se hybrid genes encode

hybrid proteins which are different from their parental natural Bacillus thuringiensis crystal toxin proteins and from any other known Bacillus thuringiensis crystal toxin protein.

Accordingly, these new hybrid proteins might have either different insecticidal activities and/or an altered insect host range as compared to the natural crystal toxin proteins produced by Bacillus thuringiensis variety kurstaki HD-1 Dipel or Bacillus thuringiensis variety kurstaki HD-73.

The experimental procedure described in the present invention in order to generate hybrid recombinants between different Bacillus thuringiensis genes can be applied to other couples of Bacillus thuringiensis genes or to different recombinant products, generating new polypeptides possibly having spectra of toxic activity different from that of the parental products. Among the 8 new different hybrid Bacillus thuringiensis crystal protein toxins isolated, preliminary results indicate that some of them have a specific toxic activity against Ostrinia nubilalis larvae (European corn borer).

It is clear to those skilled in the art that direct sequence analysis of other recombinants combined with assays of toxicity against different Lepidopteran targets could lead to the identification of new toxins with either a different insecticidal activity or an altered insect host range. Those skilled in the art will appreciate, therefore, that the invention described h rein and the methods of practising it specifically described are susceptible of variations and modifications other than as specifically

described.

It is to be und rstood that the invention includes all these variations and modifications which are intended to be fully within the scope of the following claims.

The numerals set forth below represent figure numbers for the appended drawings.

Fig. 1 - Shows the construction of pT173 plasmid.

PT is a derivative of pBS19 able to express chloramphenicol resistance (Cm) and tetracycline resistance (Tet, heavy black arrow) in <u>E. coli</u> and <u>B. subtilis</u>.

The open box is the HD-73 toxin coding sequence starting from residue 1383. The direction of transcription is indicated. The heavy line represents the HD-73 sequences downstream the toxin gene.

Dashed box represents the HD-1 Dipel sequence starting from residue 291 to residue 2215. The direction of transcription is indicated. The interrupted arrows indicate the region of partial homology.

Fig. 2 - Shows the restriction map of plasmid pGEM173 (10.8 kb)

This plasmid is obtained by inserting a truncated fragment of the HD-73 gene, more particularly an EcoRI-ecoRI fragment derived from plasmid pJWK20 (as for the construction of plasmid pT173) in the plasmid vector pGEM 4Z Promega.

The truncated HD-1 g ne inserted in plasmid pGEM-173 is a BamHI-BamHI obtained from plasmid pT173. This BamHI-BamHI fragment only includes part of the Tct^R

gene, which contains a NrvI restriction site.

Fig. 3 - Is a schematic representation of the process of in vivo recombination of the present invention.

Plasmid PT 173 (13 kb) carrying the Bacillus thuringiensis HD-1 and HD-73 truncated sequences, separated by the marker gene for the resistance to tetracycline, has been introduced by transformation in E. coli ($RecA^+$) cells wherein in vivo recombination of the two Bacillus thuringiensis DNA sequences occurs.

PTHY represents isolated recombinant vectors carrying genes coding for a new hybrid Bacillus thuringiensis toxin.

The heavy closed boxes represent the partially homologous regions, open for HD-73, dashed for HD-1 Dipel.

Fig. 4 - Shows the alignment of the two Bacillus thuringiensis DNA sequences HD-1 and HD-73 in order to give the greatest homology.

Vertical dashed lines indicate matches; boxes represent, for each recombinant obtained by the present method, regions wherein the recombination has occurred; numbers in bold above boxes represent the new recombinant obtained HY 6, HY 15, HY 107, HY 53.

DNA sequence nucleotides are indicated and numbered according to the original numeration given in the published DNA sequences HD-1 (Schnepf et al., 1985) and HD-73 (Adang et al., 1985).

Fig. 5 - Shows the optimal alignment of polypeptide deduced from the sequences of HD-1 Dipel and HD-73 genes.

PCT/EP90/01145
International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 18, line 12 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

INSTITUT PASTEUR
COLLECTION NATIONAL DE MICRO-ORGANISMES

Address of depositary institution (including postal code and country) [4]

25, RUE DU DR. ROUX
75015 PARIS

Date of deposit [5]	Accession Number [6]
June 28, 1989	

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

AUSTRALIA
JAPAN
EUROPEAN PATENT
SOVIET UNION
UNITE STATES OF AMERICA

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

12. 07. 90

AvdVelden TON V.D. VELDEN
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

In Panel A (modified after Geiser et al., 1986) is schematically represented the alignment of the entire polypeptides; a vertical line represents an unmatched residue, short bars near the horizontal lines are deletions.

Panel B represents the aminoacid sequences aligned for the region corresponding to the partial homology region, at the DNA sequence level, used for the recombination in pT173 and pGEM173.

The sequence indicated in this figure corresponds to the region underlined in Panel A.

An aminoacid residue identical in HD-1 Dipel and HD-73 or in the hybrid products is denoted by a bar.

- represents an aminoacid residue deleted to obtain maximum alignment.

Figs 6 a-e Show the DNA nucleotide sequences of hypervariable regions of hybrid Bacillus thuringiensis genes obtained by in vivo recombination according to the present invention.

The DNA sequence has been determined sperimentally on both DNA strands for all the recombinant hybrid genes obtained.

HD-1 DNA sequence is in bold letters. Numeration is as follows: Position 1 corresponds to nucleotide 1521 of the HD-1 DNA sequence (Schnepf, 1985) while the last nucleotide corresponds to nucleotide 2091 of the HD-73 DNA sequence (Adang et al., 1985).

CLAIMS

1. Hybrid DNA sequences characterized in that they are obtained by in vivo recombination of two genes coding for an insecticidal toxin protein.

2. Hybrid DNA sequences according to Claim 1, wherein said hybrid DNA sequences have been obtained by in vivo recombination of two Bacillus thuringiensis genes coding for the Bacillus thuringiensis crystal toxin protein.

3. Hybrid DNA sequences according to Claims 1-2, wherein said hybrid DNA sequences have been obtained by in vivo recombination of two Bacillus thuringiensis variety kurstaki genes coding for the Bacillus thuringiensis crystal toxin protein.

4. Hybrid DNA sequences according to Claims 1-3, wherein said DNA sequences have been obtained by in vivo recombination of the Bacillus thuringiensis variety kurstaki HD-1 Dipel gene and the Bacillus thuringiensis var. kurstaki HD-73 gene, both genes coding for the Bacillus thuringiensis crystal toxin protein.

5. Hybrid DNA sequences according to Claims 1-4 wherein said hybrid DNA sequences have been obtained by in vivo recombination of the hypervariable regions of the Bacillus thuringiensis var. kurstaki HD-1 Dipel gene and of Bacillus thuringiensis var. kurstaki HD-73 gene, both genes coding for the Bacillus thuringiensis crystal toxin protein.

6. Hybrid DNA sequence HY 5 according to Claims 1-5 characterized by an hypervariable r gion having the following nucleotide sequence:

LOCUS HY5 713 BP ENTERED 5/22/87

ORIGIN IN VIVO RECOMBINANT HD1/HD73 #5

```
  1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
 61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
241 ATGTAATACC GCCACAGAAT AACAACGTGC CACCTAGGCA AGGATTTAGT CATCGATTAA
301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
```

7. Hybrid DNA sequence HY 45 according to Claims 1-5 characterized by an hypervariable region having the following sequence:

LOCUS HY45 707 BP ENTERED 5/23/89

ORIGIN IN VIVO RECOMBINANTS HD-1/HD73 #45,66

```
  1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
 61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGC CATCGATTGA
301 GTCATGTTAC AATGCTGAGC CAAGCAGCTG GAGCAGTTTA CACCTTGAGA GCTCCAACGT
361 TTTCTTGGAT ACATCGTAGT GCTGAATTTA ATAATATAAT TGCATCGGAT AGTATTACTC
421 AAATCCCTGC AGTGAAGGGA AACTTTCTTT TTAATGGTTC TGTAATTTCA GGACCAGGAT
481 TTACTGGTGG GGACTTAGTT AGATTAAATA GTAGTGGAAA TAACATTCAG AATAGAGGGT
541 ATATTGAAGT TCCAATTCAC TTCCCATCGA CATCTACCAG ATATCGAGTT CGTGTACGGT
601 ATGCTTCTGT AACCCCGATT CACCTCAACG TTAATTGGGG TAATTCATCC ATTTTTTCCA
661 ATACAGTACC AGCTACAGCT ACGTCATTAG ATAATCTACA ATCAAGT
```

8. Hybrid DNA sequence HY 3 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY3 710BP ENTERED 5/23/89

ORIGIN IN VIVO RECOMBINANTS HD-1/HD73 #3,4,7

```
  1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
 61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGC CATCGATTGA
301 GTCATGTTAC AATGCTGAGC CAAGCAGCTG GAGCAGTTTA CACCTTGAGA GCTCCAACGT
361 TTTCTTGGCA GCATCGCAGT GCTGAATTTA ATAATATAAT TCCTTCATCA CAAATTACAC
421 AAATACCTTT AACAAAATCT ACTAATCTTG GCTCTGGAAC TTCTGTCGTT AAAGGACCAG
481 GATTTACTGG TGGGGACTTA GTTAGATTAA ATAGTAGTGG AAATAACATT CAGAATAGAG
541 GGTATATTGA AGTTCCAATT CACTTCCCAT CGACATCTAC CAGATATCGA GTTCGTGTAC
601 GGTATGCTTC TGTAACCCCG ATTCACCTCA ACGTTAATTG GGGTAATTCA TCCATTTTTT
661 CCAATACAGT ACCAGCTACA GCTACGTCAT TAGATAATCT ACAATCAAGT
```

9. Hybrid DNA sequence HY 21 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY21 713BP ENTERED 5/23/89

ORIGIN IN VIVO RECOMBINANT HD-1/HD73 #21

```
  1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
 61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGC CATCGATTGA
301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
```

10. Hybrid DNA sequence HY 32 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

```
LOCUS       HY32        707BP   ENTERED 5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #32
  1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
 61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGC CATCGATTGA
301 GTCATGTTAC AATGCTGAGC CAAGCAGCTG GAGCAGTTTA CACCTTGAGA GCTCCTATGT
361 TCTCTTGGAT ACATCGTAGT GCTGAATTTA ATAATATAAT TGCATCGGAT AGTATTACTC
421 AAATCCCTGC AGTGAAGGGA AACTTTCTTT TTAATGGTTC TGTAATTTCA GGACCAGGAT
481 TTACTGGTGG GGACTTAGTT AGATTAAATA GTAGTGGAAA TAACATTCAG AATAGAGGGT
541 ATATTGAAGT TCCAATTCAC TTCCCATCGA CATCTACCAG ATATCGAGTT CGTGTACGGT
601 ATGCTTCTGT AACCCCGATT CACCTCAACG TTAATTGGGG TAATTCATCC ATTTTTTCCA
661 ATACAGTACC AGCTACAGCT ACGTCATTAG ATAATCTACA ATCAAGT
```

11. Hybrid DNA sequence HY 6 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

```
LOCUS       HT6         710BP   UPDATED 5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #6
  1 AATTCGCATT CCCTTTATTT GGGAATGCGG GAAATGCAGC TCCACAACAA CGTATTGTTG
 61 CTCAACTAGG TCAGGGCGTG TATAGAACAT TATCGTCCAC TTTATATAGA AGACCTTTTA
121 ATATAGGGAT AAATAATCAA CAACTATCTG TTCTTGACGG GACAGAATTT GCTTATGGAA
181 CCTCCTCAAA TTTGCCATCC GCTGTATACA GAAAAAGCGG AACGGTAGAT TCGCTGGATG
241 AAATACCGCC ACAGAATAAC AACGTGCCAC CTAGGCAAGG ATTTAGTCAT CGATTAAGCC
301 ATGTTTCAAT GTTTCGTTCA GGCTTTAGTA ATAGTAGTGT AAGTATAATA AGAGCTCCTA
361 TGTTCTCTTG GATACATCGT AGTGCTGAAT TTAATAATAT AATTGCATCG GATAGTATTA
421 CTCAAATCCC TGCAGTGAAG GGAAACTTTC TTTTTAATGG TTCTGTAATT TCAGGACCAG
481 GATTTACTGG TGGGGACTTA GTTAGATTAA ATAGTAGTGG AAATAACATT CAGAATAGAG
541 GGTATATTGA AGTTCCAATT CACTTCCCAT CGACATCTAC CAGATATCGA GTTCGTGTAC
601 GGTATGCTTC TGTAACCCCG ATTCACCTCA ACGTTAATTG GGGTAATTCA TCCATTTTTT
661 CCAATACAGT ACCAGCTACA GCTACGTCAT TAGATAATCT ACAATCAAGT
```

12. Hybrid DNA sequence HY 53 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY53 710BP UPDATED 5/22/89

ORIGIN IN VIVO RECOMBINANTS HD-1/HD73

#53,64,107

```
  1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACAACAA CGTATTGTTG
 61 CTCAACTAGG TCAGGGCGTG TATAGAACAT TATCGTCCAC TTTATATAGA AGACCTTTTA
121 ATATAGGGAT AAATAATCAA CAACTATCTG TTCTTGACGG GACAGAATTT GCTTATGGAA
181 CCTCCTCAAA TTTGCCATCC GCTGTATACA GAAAAAGCGG AACGGTAGAT TCGCTGGATG
241 AAATACCGCC ACAGAATAAC AACGTGCCAC CTAGGCAAGG ATTTAGTCAT CGATTAAGCC
301 ATGTTTCAAT GTTTCGTTCA GGCTTTAGTA ATAGTAGTGT AAGTATAATA AGAGCTCCTA
361 TGTTCTCTTG GATACATCGT AGTGCTGAAT TTAATAATAT AATTGCATCG GATAGTATTA
421 CTCAAATCCC TGCAGTGAAG GGAAACTTTC TTTTTAATGG TTCTGTAATT TCAGGACCAG
481 GATTTACTGG TGGGGACTTA GTTAGATTAA ATAGTAGTGG AAATAACATT CAGAATAGAG
541 GGTATATTGA AGTTCCAATT CACTTCCCAT CGACATCTAC CAGATATCGA GTTCGTGTAC
601 GGTATGCTTC TGTAACCCCG ATTCACCTCA ACGTTAATTG GGGTAATTCA TCCATTTTTT
661 CCAATACAGT ACCAGCTACA GCTACGTCAT TAGATAATCT ACAATCAAGT
```

13. Hybrid DNA sequence HY 2 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY2 713BP UPDATED 5/23/89

ORIGIN IN VIVO RECOMBINANT HD-1/HD73 #2

```
  1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
 61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
241 ATGTAATACC GCCACAGGAT AATAGTGTGC CACCTAGGCA AGGATTTAGT CATCGATTAA
301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
```

14. Hybrid DNA sequence HY 127 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY127 713BP ENTERED 5/23/89

ORIGIN IN VIVO RECOMBINANT HD-1/HD73 #127

```
  1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
 61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGT CATCGATTAA
301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
```

15. Hybrid DNA sequence HY 126 according to Claims 1-5 characterized by an hypervariable region having the following nucleotide sequence:

LOCUS HY126 707 BP ENTERED 5/22/89

ORIGIN IN VIVO RECOMBINANT HD-1/HD 73 #126

```
  1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
 61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA CCTTTTAATA
121 TAGGGATAAA TAATCAACAA CTATCTGTTC TTGACGGGAC AGAATTTGCT TATGGAACCT
181 CCTCAAATTT GCCATCCGCT GTATACAGAA AAAGCGGAAC GGTAGATTCG CTGGATGAAA
241 TACCGCCACA GAATAACAAC GTGCCACCTA GGCAAGGATT TAGTCATCGA TTAAGCCATG
301 TTTCAATGTT TCGTTCAGGC TTTAGTAATA GTAGTGTAAG TATAATAAGA GCTCCTATGT
361 TCTCTTGGAT ACATCGTAGT GCTGAATTTA ATAATATAAT TGCATCGGAT AGTATTACTC
421 AAATCCCTGC AGTGAAGGGA AACTTTCTTT TTAATGGTTC TGTAATTTCA GGACCAGGAT
481 TTACTGGTGG GGACTTAGTT AGATTAAATA GTAGTGGAAA TAACATTCAG AATAGAGGGT
541 ATATTGAAGT TCCAATTCAC TTCCCATCGA CATCTACCAG ATATCGAGTT CGTGTACGGT
601 ATGCTTCTGT AACCCCGATT CACCTCAACG TTAATTGGGG TAATTCATCC ATTTTTTCCA
661 ATACAGTACC AGCTACAGCT ACGTCATTAG ATAATCTACA ATCAAGT
```

16. Hybrid insecticidal crystal toxin protein encoded by the hybrid DNA sequences according to Claims 1-15.

17. Hybrid insecticidal crystal toxin proteins having substantially the immunological properties of the hybrid insecticidal crystal toxin protein

according to Claim 16.

18. Muteins of the hybrid insecticidal crystal toxin prot ins according either to Claim 16 or Claim 17 wherein said muteins have been obtained by standardized genetic engineering techniques such as site-specific mutagenesis, random mutagenesis, site-specific glycosilation, and their activities are reconducible to the insecticidal activities of their parental hybrid insecticidal crystal toxin proteins according to either Claim 16 or Claim 17.

19. Plasmid vectors characterized in that they contain two genes coding for an insecticidal toxin protein and having enough residual homology to be able to recombine <u>in vivo</u>, wherein said genes upon <u>in vivo</u> recombination produce the hybrid DNA sequences of Claims 1-15.

20. Plasmid vectors according to Claim 19 further characterized in that the genes coding for the insecticidal toxin protein are accociated and separated on said plasmid vectors by a DNA fragment acting as an antibiotic resistance marker.

21. Plasmid vectors according to Claim 19 wherein the genes coding for an insecticidal toxin protein are <u>Bacillus thuringiensis</u> genes coding for the <u>Bacillus thuringiensis</u> crystal toxin protein.

22. Plasmid vectors according to Claim 21 wherein the <u>Bacillus thuringiensis</u> genes are <u>Bacillus thuringiensis</u> variety <u>kurstaki</u> genes coding for the <u>Bacillus thuringiensis</u> variety <u>kurstaki</u> crystal toxin prot in.

23. Plasmid vectors according to Claim 22 wherein the <u>Bacillus thuringiensis</u> variety <u>kurstaki</u>

genes have been derived from <u>Bacillus</u> <u>thuringiensis</u> variety <u>kurstaki</u> HD-1 Dipel and from <u>Bacillus</u> <u>thuringiensis</u> variety <u>kurstaki</u> HD 73, said genes coding both for crystal toxin protein.

24. Plasmid vectors according to Claim 23 wherein said plasmid vectors are pT173 and pGEM173.

25. Plasmid vector characterized in that it contains a first gene coding for a first insecticidal toxin protein and a second gene coding for a second insecticidal toxin protein, having enough amology in order to be able to recombine <u>in</u> <u>vivo</u>, so that it produces hybrid DNA sequences coding for a third insecticidal toxin protein, wherein said first gene is characteristic of the hypervariable region of a first bacterial strain, while the said second gene is characteristic of the hypervariable region of a second bacterial strain substantially different from the said first strain.

26. Vector according to claim 25 characterized in that said first bacterial strain and said second bacterial strain belong to the same genera, preferably <u>Bacillus</u> type.

27. Vector according to claim 26 characterized in that said first bacterial strain and said second bacterial strain belong to the same species, preferably <u>Bacillus</u> <u>thuringiensis</u> type.

28. Vector according to claim 27 characterized in that said first bacterial strain and said second bacterial strain belong to the same subspecies, preferably of the <u>Bacillus</u> <u>thuringiensis</u> <u>kurstaki</u> type.

29. Plasmid expression vectors characterized in

that they have been obtained by in vivo recombination of the two genes coding for an insecticidal toxin protein contained in plasmid vectors according to Claims 19 and 25 wherein said plasmid vectors obtained by in vivo recombination contain the hybrid DNA sequences of Claims 1-15.

30. Plasmid expression vectors containing the hybrid DNA sequences according to Claims 1-15 under the control of expression regulatory functions.

31. Plasmid expression vectors according to Claim 29 wherein said expression regulatory functions include, but are not limited to, the lac system, the Trp system, the major operator and promoter regions of phage λ, the tac system, the β-lac system.

32. Plasmid expression vectors containing the hybrid DNA sequences according to Claims 1-15 wherein said plasmid expression vectors are used to transform plant cells.

33. Plant cells transformed by plasmid expression vectors according to Claim 29.

34. Transgenic insect resistant plants containing the hybrid DNA sequences according to Claims 1-15.

35. Genetically engineered plant colonizing microorganism containing the hybrid DNA sequences according to Claim 1-15.

36. Host cells transformed by the plasmid expression vectors according to Claim 29.

37. Host cells according to Claim 36 wherein said host cells are various strains of *Bacillus*, including *B. subtilis*, *B. thuringiensis*, Yeasts, *Agrobacterium*, baculoviruses, *Rhizobium*.

38. Host cells according to Claim 36 wherein said host cells are Escherichia Coli host cells.

39. Host cells according to Claim 38 wherein said host cells can be recombinant unproficient cells rec A^-.

40. Escherichia coli host cells transformed with plasmid vector pT173.

41. Escherichia coli host cells transformed with plasmid vector pGEM 173.

42. A process for preparing the hybrid DNA sequences of Claims 1-15 by in vivo recombination of two or more genes coding for an insecticidal toxin protein and having enough residual homology to be able to recombine in vivo.

43. A process according to Claim 42, wherein said genes coding for an insecticidal protein are Bacillus thuringiensis genes coding for the Bacillus thuringiensis crystal toxin protein.

44. Pesticidal compositions and formulations containing the hybrid crystal toxin proteins of Claims 16 and 17 in combination with suitable eccipients, diluents, fillers, aggregant and the like.

45. Use of the hybrid crystal toxin proteins of Claim 17 to control and to combat insect pest.

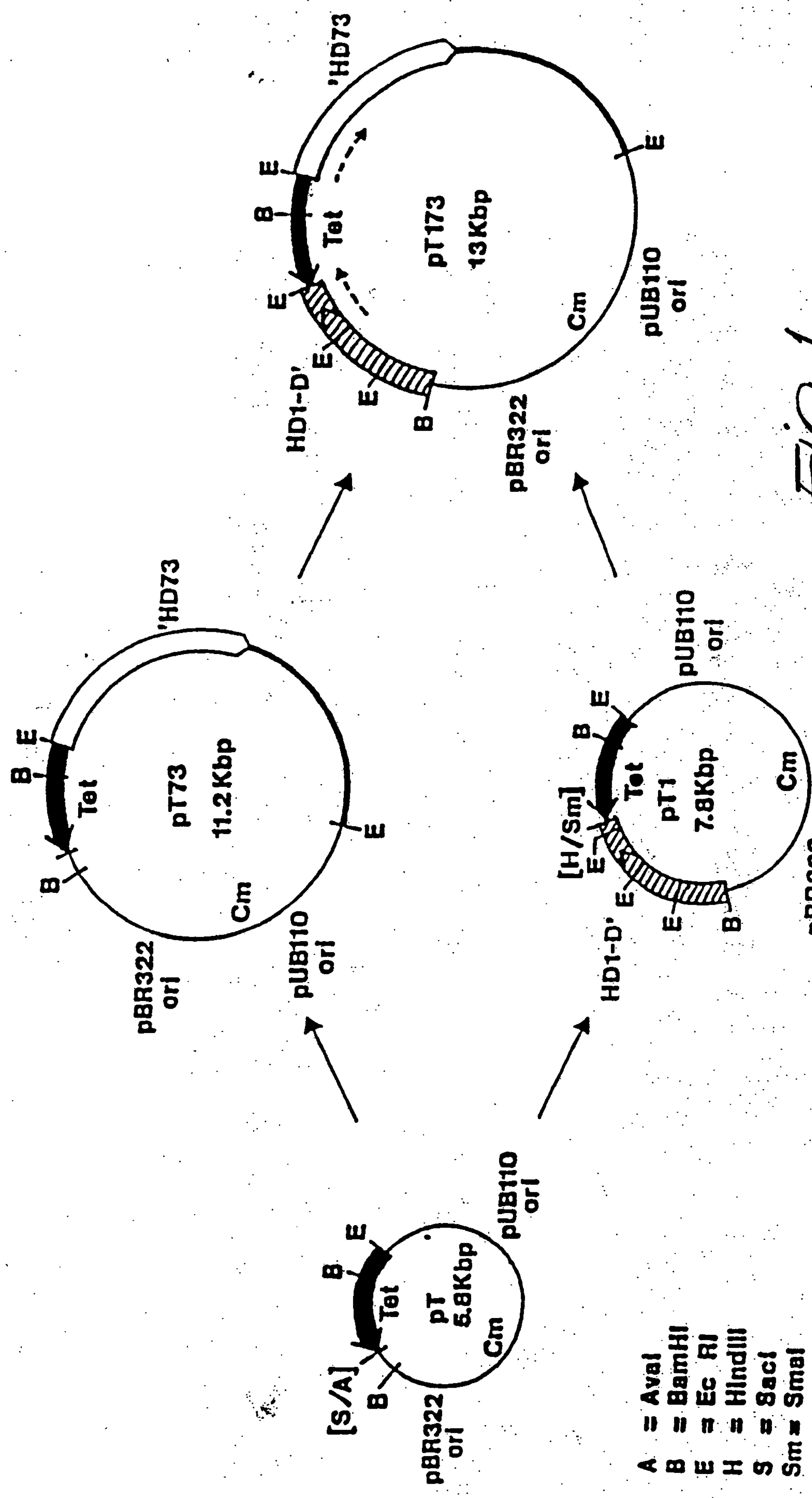

pT
5.8Kbp
Tet
Cm
[S/A]
B
E
pBR322
ori
pUB110
ori
pT73
11.2Kbp
'HD73
pT1
7.8Kbp
[H/Sm]
HD1-D'
pT173
13Kbp
A = AvaI
B = BamHI
E = Ec RI
H = HindIII
S = SacI
Sm = SmaI

Fig. 1

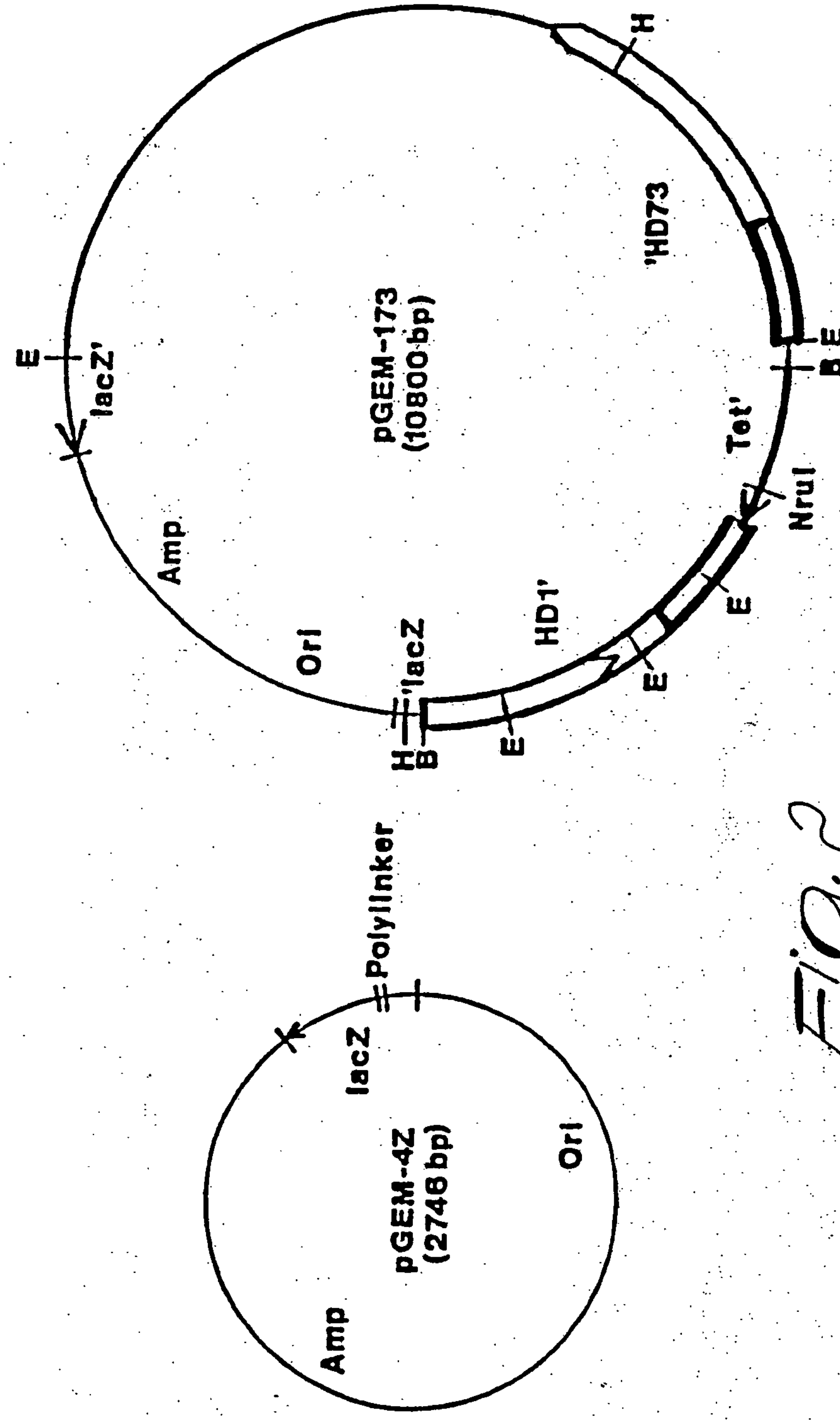

pGEM-173
(10800 bp)
'HD73
H
B E
Tet'
NruI
E
E
E
HD1'
'lacZ
H
B
Ori
Amp
lacZ'
E
Polylinker
lacZ
pGEM-4Z
(2746 bp)
Ori
Amp

Fig. 2

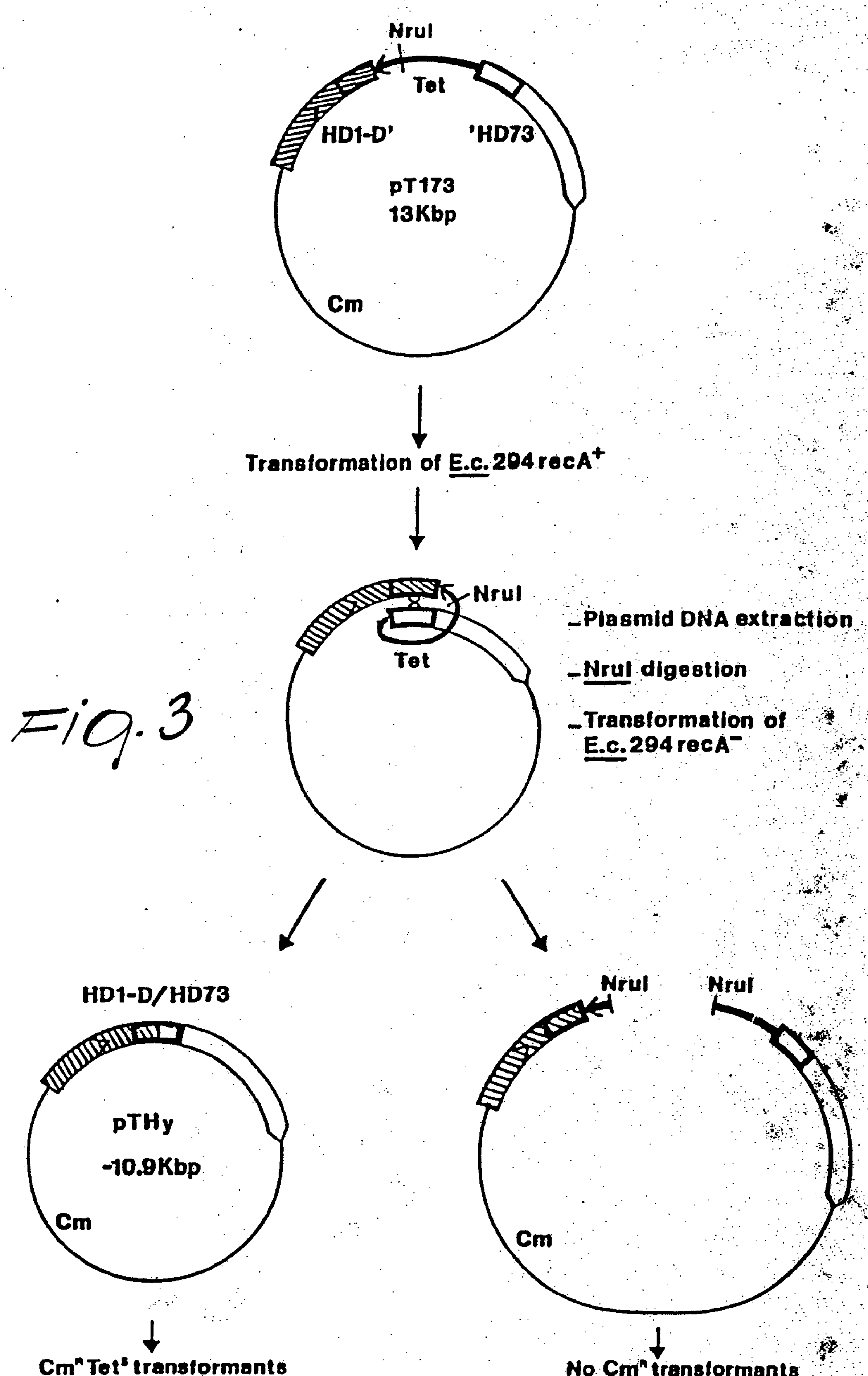
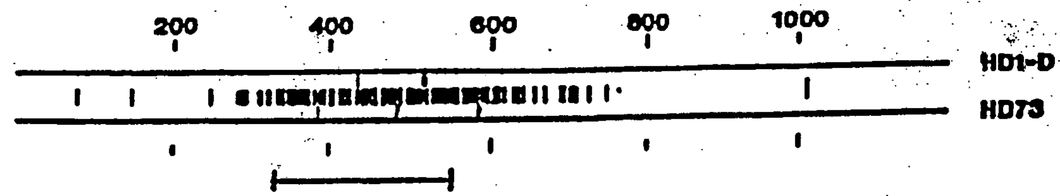

NruI
Tet
HD1-D'
'HD73
pT173
13Kbp
Cm
Transformation of E.c. 294 recA+
NruI
Tet
-Plasmid DNA extraction
-NruI digestion
-Transformation of E.c. 294 recA-
HD1-D/HD73
pTHy
~10.9Kbp
Cm
NruI
NruI
Cm
CmR TetS transformants
No CmR transformants

Fig. 3

```
HD 1 Dipel gene                                                    LIMITS: 1521
HD 73 gene                                                         LIMITS: 1383

                                          6.15  64.53.107
1521   AATTCGCATTCCCTTTATTTGGGAATGCGGGGAATGCAGCTCCACCCGTACTTGT CTC
1383    ATTCACTTTTCCGCTATATGGAACTATGGGAAATGCAGCTCCACAACAACGTATTGTTG
                                                        126
1579   ATTAACT GGTTTGGGGATTTTTAGAACATTATCTTCACCTTTATATAGAAGAATTATAC
1442   CTCAACTAGGTCAGGGCGTGTATAGAACATTATCGTCCACTTTATATAGAAGACCT T

1638   TTGGTTCAGGCCCAAATAATCAGGAACTGTTTGTCCTTGATGGAACGGAGTTTTCTTTTG
1499   TTAATATAGGGATAAATAATCAACAACTATCTGTTCTTGACGGGACAGAATTTGCTTATG

1698   CCTCCCTAACGACCAACTTGCCTTCCACTATATATAGACAAAGGGGTACAGTCGATTCAC
1559    GAACCT   CCTCAAATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCGC
               5                    2               127
1758   TAGATGTAATACCGCCACAGGATAATAGTGTACCACCTCGTGCGGGATTTAGCCATCGAT
1616   TGGATGAAATACCGCCACAGAATAACAACGTGCCACCTAGGCAAGGATTTAGTCATCGAT
        21                                                       32
1818   TGAGTCATGTTACAATGCTGAG CCAAGC    AGCTGGAGCAGTTTA  CACCTTGAGAG
1676   TAAGCCATGTTTCAATGTTTCGTTCAGGCTTTAGTAATAGTAGTGTAAGTATAATAAGAG
                66.45
1872   CTCCAACGTTTTCTTGGCAGCATCGCAGTGCTGAATTTAATAATATAATTCCTTCATCAC
1736   CTCCTATGTTCTCTTGGATACATCGTAGTGCTGAATTTAATAATATAATTGCATCGGATA

1932   AAATTACACAAATACCTTTAACAAAATCTACTAATCTTGGCTCTGGAACTTCTGTCGTTA
1796   GTATTACTCAAATCCCTGCAGTGAAGGGAAACTTTCTTTTTAATGG   TTCTGTAATTT
            3.4.7
1992   AAGGACCAGGATTTACAGGAGGAGATATTCTTCGAAGAACTTCACCTGGCCAGA  TTTC
1853   CAGGACCAGGATTTACTGGTGGGGACTTAGTTAGATTAAATAGTAGTGGAAATAACATTC

2050   AACCTTAAGAGTAAAT  A  TT  ACTGCA   CCAT  TATCACAAAGATATCGGG
1913   AGAATAGAGGGTATATTGAAGTTCCAATTCACTTCCCATCGACATCTACCAGATATCGAG

2097   TAAGAATTCGCTACGCTTCTACTACAAATTTACAATTCCATACATCAATTGACGGAA  G
1973   TTCGTGTACGGTATGCTTCTGTAACCCCGATTCACCT CA ACGTTAATTGGGGTAATTC

2155   ACCTATTAATCAGGGTAATTTTTCAGCAAC TATGAGTAGTGGGAGTAATTTACAGTCCG
2031   ATCCATTTTTTCCAATACAGTACCAGCTACAGCTACGTCATTAGA TAATCTACAATCAA

2214   GA
2090   GT

Matches = 456     Mismatches = 226     Unmatched = 40
Length = 722      Matches/length =  63.2 percent
```

FIG. 4



B

HYBRID PROTEINS FROM RECOMBINANT GENES

```
HD1-D          333  FAFPLFSNASNAAPPV^LVSLTSLSIFRTLSSPLYRRIILSSSPNNQELFVLDGTEFSFAS
HY7,3,4             ------------------------------------------------------------
HY66,45             ------------------------------------------------------------
HY32                ------------------------------------------------------------
HY127,21            ------------------------------------------------------------
HY2                 ------------------------------------------------------------
HY5                 ------------------------------------------------------------
HY126               ------------------------------------P^FNI-I---Q-S-------AYGT
HY6,53,64,107       ---------------QQRI-AQL-Q-VY-----T----P^FNI-I---Q-S-------AYGT
HY15                -----------KCSSTTTYCCSTRSGRV*
HD73                -T---Y-TH-------QQRI-AQL-Q-VY-----T----P^FNI-I---Q-S-------AYGT

HD1-D          393  LTTNLPSTIYRQRSTVDSLDVIPPQDNSVPPRAGFSHRLSHVTM^LSQA^AGAVYTLRAPT
HY7,3,4             ------------------------------------------------------------
HY66,45             ------------------------------------------------------------
HY32-               -----------------------------------------------------------M
HY127,21            ------------------------------------------S-FR-GFSNSS-SII---M
HY2                 -------------------------------Q----------S-FR-GFSNSS-SII---M
HY5                 -------------------------N-N----Q---------S-FR-GFSNSS-SII---M
HY126               ^SS----AV--KS-------E----N-N----Q---------S-FR-GFSNSS-SII---M
HY6,53,64,107       ^SS----AV--KS-------E----N-N----Q---------S-FR-GFSNSS-SII---M
HD73                ^SS----AV--KS-------E----N-N----Q---------S-FR-GFSNSS-SII---M

HD1-D          452  FSWQHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDILRRTSPGQISTLRV
HY7,3,4             ------------------------------------------LV-LN-S-NNIQN-G
HY66,45             ---I-----------A-DS-----AV-GNFLFN-^--IS---------LV-LN-S-NNIQN-G
HY32                ---I-----------A-DS-----AV-GNFLFN-^--IS---------LV-LN-S-NNIQN-G
HY127,21            ---I-----------A-DS-----AV-GNFLFN-^--IS---------LV-LN-S-NNIQN-G
HY2                 ---I-----------A-DS-----AV-GNFLFN-^--IS---------LV-LN-S-NNIQN-G
HY5                 ---I-----------A-DS-----AV-GNFLFN-^--IS---------LV-LN-S-NNIQN-G
HY126               ---I-----------A-DS-----AV-GNFLFN-^--IS---------LV-LN-S-NNIQN-G
HY6,53,64,107       ---I-----------A-DS-----AV-GNFLFN-^--IS---------LV-LN-S-NNIQN-G
HD73                ---I-----------A-DS-----AV-GNFLFN-^--IS---------LV-LN-S-NNIQN-G

HD1-D          513  NITAPLSQRYRV^RIRYASTTNLQFHTSIDGRPINQGNF^SATMSSGSNLQSGS 564
HY7,3,4             Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY66,45             Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY32                Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY127,21            Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY2                 Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY5                 Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY126               Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HY6,53,64,107       Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
HD73                Y-EV-IHFPSTST-Y-VRVRYASVTPIHLNVNWG-SSI-SNTVPATATS-DNLQ
```

Fig. 5

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LOCUS       HY45              707 BP                       ENTERED  5/23/89
ORIGIN      IN VIVO RECOMBINANTS HD-1/HD73 #45,66
        1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
       61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
      121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
      181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
      241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGC CATCGATTGA
      301 GTCATGTTAC AATGCTGAGC CAAGCAGCTG GAGCAGTTTA CACCTTGAGA GCTCCAACGT
      361 TTTCTTGGAT ACATCGTAGT GCTGAATTTA ATAATATAAT TGCATCGGAT AGTATTACTG
      421 AAATCCCTGC AGTGAAGGGA AACTTTCTTT TTAATGGTTC TGTAATTTCA GGACCAGGAT
      481 TTACTGGTGG GGACTTAGTT AGATTAAATA GTAGTGGAAA TAACATTCAG AATAGAGGGT
      541 ATATTGAAGT TCCAATTCAC TTCCCATCGA CATCTACCAG ATATCGAGTT CGTGTACGGT
      601 ATGCTTCTGT AACCCCGATT CACCTCAACG TTAATTGGGG TAATTCATCC ATTTTTTCCA
      661 ATACAGTACC AGCTACAGCT ACGTCATTAG ATAATCTACA ATCAAGT
//

LOCUS       HY3               710 BP                       ENTERED  5/23/89
ORIGIN      IN VIVO RECOMBINANTS HD-1/HD73 #3,4,7
        1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
       61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
      121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
      181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
      241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGC CATCGATTGA
      301 GTCATGTTAC AATGCTGAGC CAAGCAGCTG GAGCAGTTTA CACCTTGAGA GCTCCAACGT
      361 TTTCTTGGCA GCATCGCAGT GCTGAATTTA ATAATATAAT TCCTTCATCA CAAATTACAC
      421 AAATACCTTT AACAAAATCT ACTAATCTTG GCTCTGGAAC TTCTGTCGTT AAAGGACCAG
      481 GATTTACTGG TGGGGACTTA GTTAGATTAA ATAGTAGTGG AAATAACATT CAGAATAGAG
      541 GGTATATTGA AGTTCCAATT CACTTCCCAT CGACATCTAC CAGATATCGA GTTCGTGTAC
      601 GGTATGCTTC TGTAACCCCG ATTCACCTCA ACGTTAATTG GGGTAATTCA TCCATTTTTT
      661 CCAATACAGT ACCAGCTACA GCTACGTCAT TAGATAATCT ACAATCAAGT
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Fig. 6a

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LOCUS       HY6           710 BP                       UPDATED  5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #6
        1 AATTCGCATT CCCTTTATTT GGGAATGCGG GAAATGCAGC TCCACAACAA CGTATTGTTG
       61 CTCAACTAGG TCAGGGCGTG TATAGAACAT TATCGTCCAC TTTATATAGA AGACCTTTTA
      121 ATATAGGGAT AAATAATCAA CAACTATCTG TTCTTGACGG GACAGAATTT GCTTATGGAA
      181 CCTCCTCAAA TTTGCCATCC GCTGTATACA GAAAAAGCGG AACGGTAGAT TCGCTGGATG
      241 AAATACCGCC ACAGAATAAC AACGTGCCAC CTAGGCAAGG ATTTAGTCAT CGATTAAGCC
      301 ATGTTTCAAT GTTTCGTTCA GGCTTTAGTA ATAGTAGTGT AAGTATAATA AGAGCTCCTA
      361 TGTTCTCTTG GATACATCGT AGTGCTGAAT TTAATAATAT AATTGCATCG GATAGTATTA
      421 CTCAAATCCC TGCAGTGAAG GGAAACTTTC TTTTTAATGG TTCTGTAATT TCAGGACCAG
      481 GATTTACTGG TGGGGACTTA GTTAGATTAA ATAGTAGTGG AAATAACATT CAGAATAGAG
      541 GGTATATTGA AGTTCCAATT CACTTCCCAT CGACATCTAC CAGATATCGA GTTCGTGTAC
      601 GGTATGCTTC TGTAACCCCG ATTCACCTCA ACGTTAATTG GGGTAATTCA TCCATTTTTT
      661 CCAATACAGT ACCAGCTACA GCTACGTCAT TAGATAATCT ACAATCAAGT
//

LOCUS       HY53          710 BP                       UPDATED  5/22/89
ORIGIN      IN VIVO RECOMBINANTS HD-1/HD73 #53,64,107
        1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACAACAA CGTATTGTTG
       61 CTCAACTAGG TCAGGGCGTG TATAGAACAT TATCGTCCAC TTTATATAGA AGACCTTTTA
      121 ATATAGGGAT AAATAATCAA CAACTATCTG TTCTTGACGG GACAGAATTT GCTTATGGAA
      181 CCTCCTCAAA TTTGCCATCC GCTGTATACA GAAAAAGCGG AACGGTAGAT TCGCTGGATG
      241 AAATACCGCC ACAGAATAAC AACGTGCCAC CTAGGCAAGG ATTTAGTCAT CGATTAAGCC
      301 ATGTTTCAAT GTTTCGTTCA GGCTTTAGTA ATAGTAGTGT AAGTATAATA AGAGCTCCTA
      361 TGTTCTCTTG GATACATCGT AGTGCTGAAT TTAATAATAT AATTGCATCG GATAGTATTA
      421 CTCAAATCCC TGCAGTGAAG GGAAACTTTC TTTTTAATGG TTCTGTAATT TCAGGACCAG
      481 GATTTACTGG TGGGGACTTA GTTAGATTAA ATAGTAGTGG AAATAACATT CAGAATAGAG
      541 GGTATATTGA AGTTCCAATT CACTTCCCAT CGACATCTAC CAGATATCGA GTTCGTGTAC
      601 GGTATGCTTC TGTAACCCCG ATTCACCTCA ACGTTAATTG GGGTAATTCA TCCATTTTTT
      661 CCAATACAGT ACCAGCTACA GCTACGTCAT TAGATAATCT ACAATCAAGT
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Fig. 6b

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LOCUS       HY21          713 BP                          ENTERED  5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #21
        1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
       61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
      121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
      181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
      241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGC CATCGATTGA
      301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
      361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
      421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
      481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
      541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
      601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
      661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
//

LOCUS       HY32          707 BP                          ENTERED  5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #32
        1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
       61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
      121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
      181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
      241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGC CATCGATTGA
      301 GTCATGTTAC AATGCTGAGC CAAGCAGCTG GAGCAGTTTA CACCTTGAGA GCTCCTATGT
      361 TCTCTTGGAT ACATCGTAGT GCTGAATTTA ATAATATAAT TGCATCGGAT AGTATTACTC
      421 AAATCCCTGC AGTGAAGGGA AACTTTCTTT TTAATGGTTC TGTAATTTCA GGACCAGGAT
      481 TTACTGGTGG GGACTTAGTT AGATTAAATA GTAGTGGAAA TAACATTCAG AATAGAGGGT
      541 ATATTGAAGT TCCAATTCAC TTCCCATCGA CATCTACCAG ATATCGAGTT CGTGTACGGT
      601 ATGCTTCTGT AACCCCGATT CACCTCAACG TTAATTGGGG TAATTCATCC ATTTTTTCCA
      661 ATACAGTACC AGCTACAGCT ACGTCATTAG ATAATCTACA ATCAAGT
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Fig. 6c

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LOCUS       HY126          707 BP                          ENTERED  5/22/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #126
        1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTGAT
       61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA CCTTTTAATA
      121 TAGGGATAAA TAATCAACAA CTATCTGTTC TTGACGGGAC AGAATTTGCT TATGGAACCT
      181 CCTCAAATTT GCCATCCGCT GTATACAGAA AAAGCGGAAC GGTAGATTCG CTGGATGAAA
      241 TACCGCCACA GAATAACAAC GTGCCACCTA GGCAAGGATT TAGTCATCGA TTAAGCCATG
      301 TTTCAATGTT TCGTTCAGGC TTTAGTAATA GTAGTGTAAG TATAATAAGA GCTCCTATGT
      361 TCTCTTGGAT ACATCGTAGT GCTGAATTTA ATAATATAAT TGCATCGGAT AGTATTACTC
      421 AAATCCCTGC AGTGAAGGGA AACTTTCTTT TTAATGGTTC TGTAATTTCA GGACCAGGAT
      481 TTACTGGTGG GGACTTAGTT AGATTAAATA GTAGTGGAAA TAACATTCAG AATAGAGGGT
      541 ATATTGAAGT TCCAATTCAC TTCCCATCGA CATCTACCAG ATATCGAGTT CGTGTACGGT
      601 ATGCTTCTGT AACCCCGATT CACCTCAACG TTAATTGGGG TAATTCATCC ATTTTTTCCA
      661 ATACAGTACC AGCTACAGCT ACGTCATTAG ATAATCTACA ATCAAGT
//

LOCUS       HY5            713 BP                          ENTERED  5/22/89
ORIGIN      IN VIVO RECOMBINANT HD1/HD73 #5
        1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
       61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
      121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
      181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
      241 ATGTAATACC GCCACAGAAT AACAACGTGC CACCTAGGCA AGGATTTAGT CATCGATTAA
      301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
      361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
      421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
      481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
      541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
      601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
      661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
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FIG. 6d

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LOCUS       HY2          713 BP                    UPDATED  5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #2
      1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
     61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
    121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
    181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
    241 ATGTAATACC GCCACAGGAT AATAGTGTGC CACCTAGGCA AGGATTTAGT CATCGATTAA
    301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
    361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
    421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
    481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
    541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
    601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
    661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
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LOCUS       HY127        713 BP                    ENTERED  5/23/89
ORIGIN      IN VIVO RECOMBINANT HD-1/HD73 #127
      1 AATTCGCATT CCCTTTATTT GGGAATGCGG GGAATGCAGC TCCACCCGTA CTTGTCTCAT
     61 TAACTGGTTT GGGGATTTTT AGAACATTAT CTTCACCTTT ATATAGAAGA ATTATACTTG
    121 GTTCAGGCCC AAATAATCAG GAACTGTTTG TCCTTGATGG AACGGAGTTT TCTTTTGCCT
    181 CCCTAACGAC CAACTTGCCT TCCACTATAT ATAGACAAAG GGGTACAGTC GATTCACTAG
    241 ATGTAATACC GCCACAGGAT AATAGTGTAC CACCTCGTGC GGGATTTAGT CATCGATTAA
    301 GCCATGTTTC AATGTTTCGT TCAGGCTTTA GTAATAGTAG TGTAAGTATA ATAAGAGCTC
    361 CTATGTTCTC TTGGATACAT CGTAGTGCTG AATTTAATAA TATAATTGCA TCGGATAGTA
    421 TTACTCAAAT CCCTGCAGTG AAGGGAAACT TTCTTTTTAA TGGTTCTGTA ATTTCAGGAC
    481 CAGGATTTAC TGGTGGGGAC TTAGTTAGAT TAAATAGTAG TGGAAATAAC ATTCAGAATA
    541 GAGGGTATAT TGAAGTTCCA ATTCACTTCC CATCGACATC TACCAGATAT CGAGTTCGTG
    601 TACGGTATGC TTCTGTAACC CCGATTCACC TCAACGTTAA TTGGGGTAAT TCATCCATTT
    661 TTTCCAATAC AGTACCAGCT ACAGCTACGT CATTAGATAA TCTACAATCA AGT
/
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Fig. 6e

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/01145

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A01N63/02 ; C12N15/32 ; C12P21/02 ; //C12N15/62

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	EP,A,141484 (BIOGEN N.V.) see page 8, line 18 - page 11, line 10; figures 1, 4 see page 12, line 22 - page 13, line 12 (cited in the application)	1-5, 18-19, 21-23, 25-39 42-45
Y	EP,A,228838 (MYCOGEN CORP.) see page 2, lines 15 - 22; claims see page 7; examples 3-5 (cited in the application)	1-5, 18-19, 21-23, 25-39 42-45

-/--

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 OCTOBER 1990	30. 10. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, June 1989, WASHINGTON US pages 4037 - 4041; GE, A.Z. et al.: "Location of the Bombyx mori specificity domain on a Bacillus thuringiensis delta-endotoxin protein" see the whole document	1-5, 18-19, 21-23, 25-39
		42-45
Y	NUCLEIC ACIDS RESEARCH. vol. 11, no. 16, 1983, ARLINGTON, VIRGINIA pages 5661 - 5669; WEBER,H. & WEISSMANN,C.: "Formation of genes coding for hybrid proteins by recombination between related, cloned genes in E.coli" see the whole document	1-5, 18-19, 21-23, 25-39
	(cited in the application)	42-45

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9001145
SA 38941

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 17/10/90

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-141484	15-05-85	JP-A- 60070083	20-04-85
EP-A-228838	15-07-87	JP-A- 62143689	26-06-87

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82